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14. ABSTRACT

According to the recent cancer stem cell (CSC) theory, recurrent tumor must arise from a dormant tumor stem cell whose re-growth is triggered by shifting of microenvironment. This project aims at clarifying the roles of TAM in recurrent growth of dormant stem cell in breast cancer. We hypothesize that the balance of dormancy and recurrence is determined by the ability of the tumor stem cells to recruit TAM which in turn promotes self-renewal of the stem cell. We have established necessary mouse colonies and also developed the method to generate TAM. We have also shown that TAM indeed promoted the growth of CSCs in our animal model. Due to the relocation of our entire lab, Task 2 which involves extensive animal breeding has been delayed; however, we have compensated the lack of progress in Task 2 by further extending our mechanistic study in Task 1. We have shown that the interaction of TAM with CSCs lead to enhanced secretion of PDGF-BB from TAMs which then activated stromal cells and enhanced CSC self-renewal. We believe that the outcome of our results set a new paradigm in our understanding of metastatic niche of CSCs in the bone, which may provide novel targets such as TAM, PDGF and FGF, for the treatment of recurrent disease.

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Breast cancer, dormancy, tumor associated macrophage, tumor stem cell

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INTRODUCTION

Tumor cells often induce inflammatory responses and mobilize a variety of leukocytes around the tumor region, which is considered as a normal host-defense mechanism to block cancer growth. Interestingly, the risk of breast cancer recurrence has been shown to be significantly decreased by taking anti-inflammatory drugs, suggesting that inflammation plays a critical role in recurrence (1). Although TAM is one of these inflammatory responding cells, it closely resembles M2 (alternative) phenotype which exhibits anti-inflammatory and tumor-promoting functions (2). It has been also suggested that TAM provides favorable microenvironment for tumor stem cell growth by generating a suitable niche (3). According to the recent cancer stem cell theory, which still remains a hypotheses, recurrent tumor must arise from a dormant tumor stem cell whose re-growth is triggered by shifting of microenvironment. This project aims at clarifying the roles of TAM in recurrent growth of dormant stem cell in breast cancer. We hypothesize that the balance of dormancy and recurrence is determined by the ability of the tumor stem cells to recruit TAM which in turn promotes self-renewal of the stem cell.

BODY

Task 1. To examine whether reciprocal interaction of tumor stem cells and tumor-associated macrophage (TAM) can promote the stem cell growth
(a) Isolate tumor stem cell from primary and recurrent cancer in recurrent mouse model
(b) Test whether conditioned media of tumor stem cells activates macrophage
(c) Examine whether TAM promotes growth of tumor stem cells

To accomplish Task 1(a), we have obtained MMTV-rtTA and TetO-ErbB2 mice and established both the colonies of MMTV-rtTA+/- and TetO-ErbB2+/- . We have also cross-bred these animals and
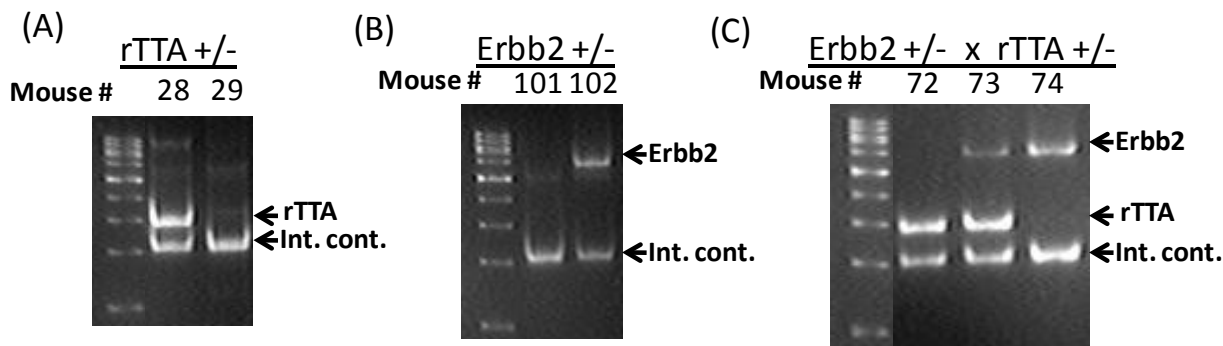

Fig. 1. Ongoing mouse colony breeding. (A) Example of genotypes of ongoing colony breeding for MMTV-rtTA. #28 mouse has rtTA transgene. (B) Example of genotypes of ongoing colony breeding for TetO-ErbB2. #102 mouse has ErbB2 transgene. (C) Example of genotypes of ongoing colony breeding for the hybrid of MMTV-rtTA and TetO-ErbB2. #72 has only rtTA, #73 has only ErbB2 and #74 has both rtTA and ErbB2 transgenes.

obtained MMTV-rtTA+/- /TetO-ErbB2+/- heterozygous animals (**Fig. 1**). These mice were not lethal without feeding Doxocycline, and they can serve for our experiments. After feeding Doxocycline, we have obtained tumors from these mice and prepared for CSCs using the established markers (Lin-/CD29+/CD24+) by the magnetic activating cell sorting (MACS) system. We also isolated CSCs population from human cancer cell line MDA-MB231BoM, which preferentially metastasizes to the bone in animal, using the CSC markers (CD24-/CD44+/ESA+) as shown in **Fig. 2**. We then tested these cells for tumor initiating abilities by the limited dilution analysis in nude mice. As shown in

Table I, these CSCs showed significantly stronger ability of tumor initiation and metastasis. Similar results were obtained for CSCs isolated from tumors in MMTV-rtTA+/- /TetO-ErbB2+/- heterozygous animals.
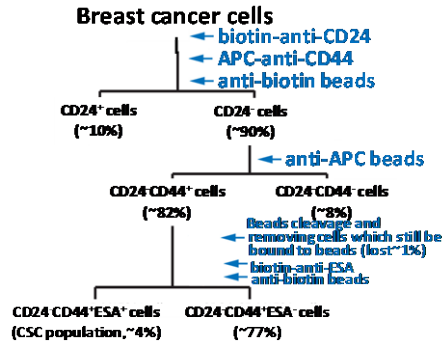
Table 1. Limiting dilution analysis for CSC

Strain	Population	Number of tumors/# of injection				CSC frequency (95% CI)
		cells/injection				
		10,000	1,000	100	10	
231BoM	Unsorted	6/6	6/7	3/5	0/3	1/334 (1/183-1/1,97)
	Stem cells	5/5	11/11	9/11	6/11	1/37** (1/19-1/72)
	Non-stem	–	2/4	0/4	0/4	1/1,671 (1/419-1/6668)

Strain	Population	# of metastasis/# of injection			Metastasis initiating cell frequency (95% confidence interval)
		Cells per injection			
		100,000	10,000	1,000	
231BoM	Stem cells	6/6	4/5	1/5	1/5,802 (1/2,182-1/15,427)
	Non-stem cell	2/6	0/5	0/5	1/274,471 (1/268,859-1/1,094,044)

*: P<0.001 (Stem cells vs Non-stem cells)

Fig. 2. Schematic diagram for the sorting procedure of CSCs.

In Aim 1(b), to establish the method for generating tumor associated macrophage (TAM), we have isolated human monocytes and treated them with IL4, IL13 and conditioned medium of human breast cancer cell, MDA-MB231BoM, which preferentially metastasizes to the bone in our animal model. As shown in **Fig. 3**, this treatment of monocytes with these three components made significant changes to the morphology of macrophages and became adherent to plastic dish.
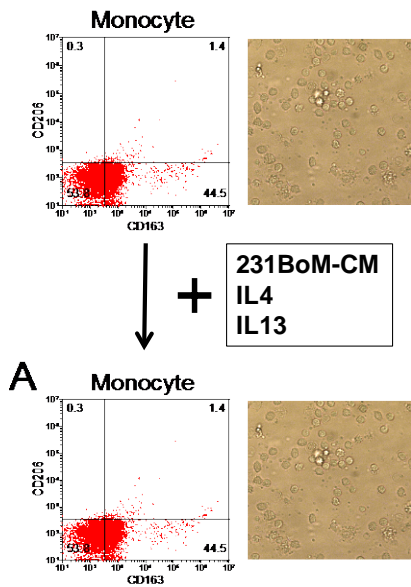

To accomplish Task 1(c), we co-injected TAM and CSCs of 231BoM directly into the tibial bones of mice followed by measuring bioluminescence for tumor growth. As shown in **Fig. 4**, the co-injection of TAMs with CSCs significantly augmented the tumor growth in the tibiae compared to the injection of CSCs alone, which strongly supports the idea that TAM plays a critical role in growth of CSCs in breast cancer.

Fig. 3. Preparation of TAM from monocytes. Primary human monocytes were treated with IL-4, IL-13 and conditioned medium of 231BoM for 7 days. A portion of the cells was analyzed by FACS using anti-CD163 and anti-CD206 antibodies. The morphology of the cells at day 7 is also shown.
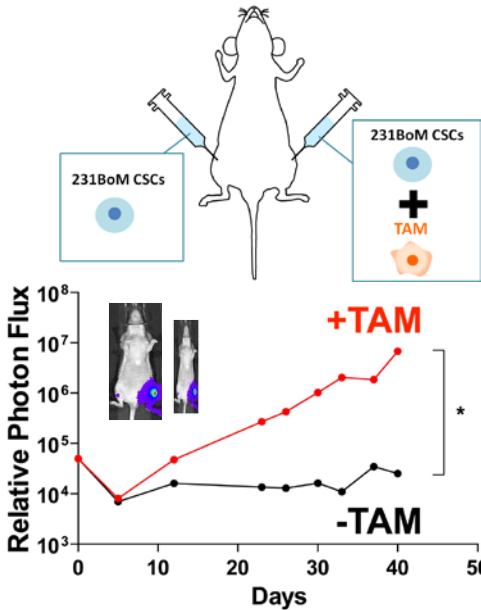
Fig. 4. Effect of TAM on the growth of CSC. CSCs from 231BoM were co-injected with (right tibia) or without (left tibia) TAMs in the same animals. The growth of tumor was then periodically measured by BLI.
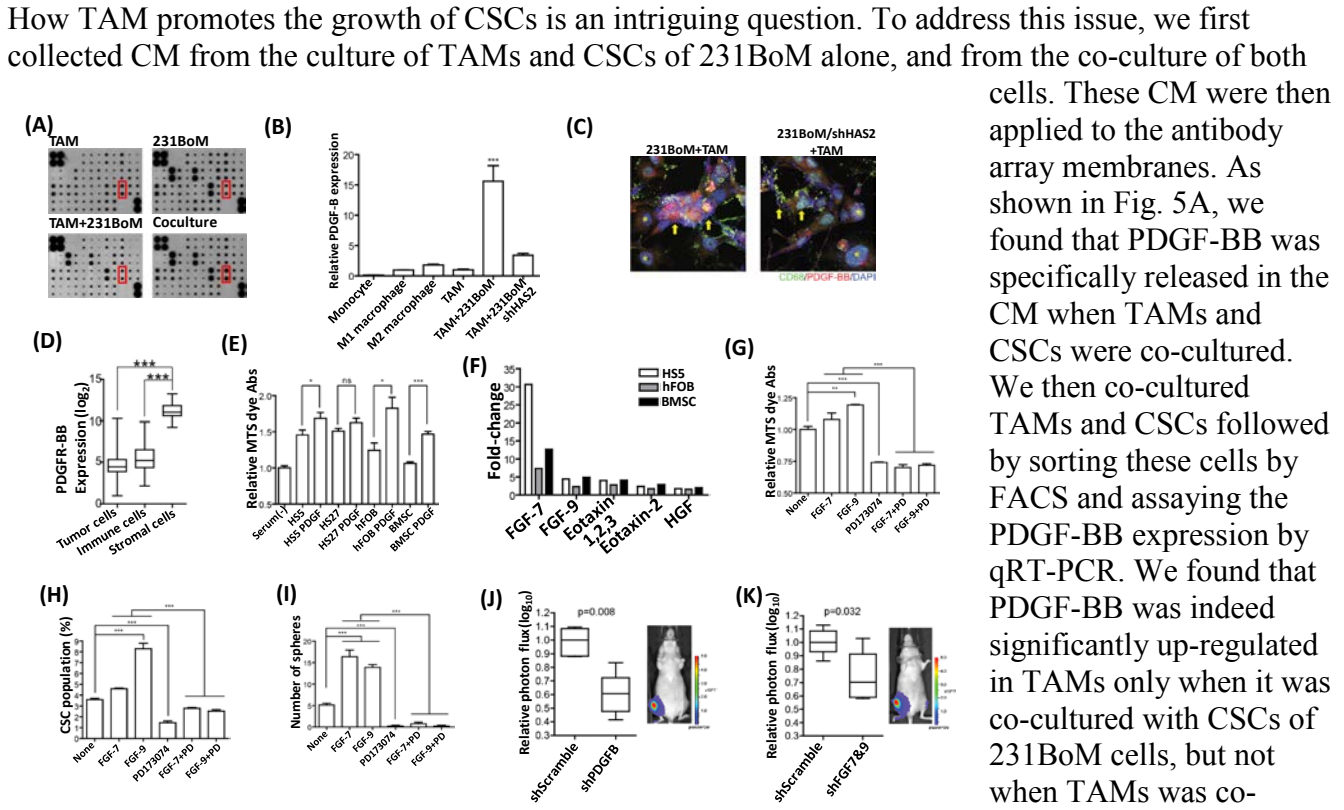
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How TAM promotes the growth of CSCs is an intriguing question. To address this issue, we first collected CM from the culture of TAMs and CSCs of 231BoM alone, and from the co-culture of both cells. These CM were then applied to the antibody array membranes. As shown in Fig. 5A, we found that PDGF-BB was specifically released in the CM when TAMs and CSCs were co-cultured. We then co-cultured TAMs and CSCs followed by sorting these cells by FACS and assaying the PDGF-BB expression by qRT-PCR. We found that PDGF-BB was indeed significantly up-regulated in TAMs only when it was co-cultured with CSCs of 231BoM cells, but not when TAMs was co-

Fig. 5. TAMs promote the growth of CSCs through activation of bone stromal cells. (**A**) CM from co-culture of TAMs and CSCs were subjected to Growth factor antibody array analysis. The position of PDGF-BB is indicated by a red box. (**B**) TAMs were co-cultured with CSCs from 231BoM for 2 days. TAMs were then sorted by FACS, and the expression of *PDGF-B* gene was quantified by qRT-PCR (n=3). (**C**) Immunofluorescent image of co-culture of TAMs with CSCs of 231BoM. (**D**) Expression levels of PDGFR-B (PDGF receptor beta) in tumor, immune and stromal cells were evaluated. The expression data of PDGFR-B were obtained from the GEO site, and they were normalized and plotted. "Tumor cells" indicates breast cancer cell lines. "Immune cells" indicates various immune cells from human blood. "Stromal cells" indicates compilation of data for fibroblasts, osteoblasts and mesenchymal stem cells. P value is calculated by Kruskal-Wallis test. *** indicates P<0.0001. (**E**) CSCs of 231BoM were treated with CM from various stromal cells that were pretreated with or without PDGF-BB (100 ng/ml) followed by measuring the growth of CSCs by the MTS assay (n=6). (**F**) CM from HS5, hFOB1.19, and BM-hMSC treated with 100 ng/mL of PDGF-BB were individually subjected to cytokine antibody array analysis. (**G**) Growth rate of CSCs of 231BoM treated with FGF9 or FGF7 and a combination with FGFR inhibitor (PD173074) was measured by the MTS assay (n=3). (**H**) CSC population of 231BoM cells by the same treatment as described in (G) was measured by FACS (n=3). (**I**) Sphere formation in suspension culture of MCF7-BoM2d cells was measured as the average number of spheres per 500 cells and the results were plotted (n=6). (**J**) CSCs of 231BoM were co-injected with TAMs/sh-scramble (left tibia) or TAMs/shPDGFB (right tibia) in the same animals (n=5). (**K**) CSCs of 231BoM were co-injected with BM-hMSC/sh-scramble (left tibia) or BM-hMSC/shFGF7&shFGF9 (right tibia) in the same animals (n=5). The normalized bioluminescent values were represented.

cultured with CSCs from 231BoM-shHAS2 (Fig. 5B). We also performed immunofluorescence analysis for the co-culture and found that PDGF-BB was expressed in TAMs specifically when they adhered with CSCs (Fig. 5C). These results indicate that PDGF-BB is specifically expressed and released from TAMs when it directly interacts with CSCs and that blocking the HAS2 expression in

CSCs significantly abrogates the induction of PDGF-BB in TAMs. We then tested a possibility that PDGF-BB released from TAMs directly stimulates the proliferation of CSCs by treating CSCs with PDGF-BB; however, the PDGF-BB treatment did not show any significant effect on the growth of CSCs (not shown). Thus, the effect of PDGF-BB on CSC, if any, is likely to be indirect. We then analyzed the expression profile of PDGFR-beta to which PDGF-BB has the highest affinity, in various types of cells using GEO database, and found that PDGFR-beta is most highly expressed in the stromal cells (Fig. 5D). Therefore, we hypothesized that PDGF-BB from TAMs indirectly affect CSCs through activation of stromal cells. To test this hypothesis, we treated the bone marrow derived fibroblasts (HS5 and HS27A), osteoblast (hFOB1.19) and bone marrow derived human mesenchymal stem cell (BM-hMSC) with PDGF-BB, and collected their CM. We then added the CM to CSCs of 231BoM followed by measuring cell proliferation and found that CM from PDGF-BB-treated stromal cells significantly enhanced the proliferation of CSCs, suggesting that PDGF-BB-activated the stromal cells to secrete a factor which in turn stimulates the CSCs proliferation. To further confirm this notion, we co-cultured TAMs and CSCs followed by collecting CM. The stromal cells were then treated with the CM and the secondary CM was collected. CSCs were then cultured with the secondary CM followed by assaying for proliferation. As shown in Fig. 5E, we found that this secondary CM significantly enhanced the proliferation of CSCs, suggesting that the interaction of CSCs and TAM secretes PDGF-BB which then activates stromal cells to secrete growth stimulating factor(s) for CSCs. To identify such factor(s), we collected CM from stromal cells after treating them with PDGF-BB followed by cytokine antibody array analysis. We found that FGF7, FGF9, eotaxins and HGF were specifically secreted in the CM of three different cells when they were treated with PDGF-BB (Fig. 5F). Among these factors, FGF7 and FGF9 are known to play important roles in embryonic development as well as in expansion and maintenance of CSCs population in breast cancers. Therefore, we examined the effects of these factors on CSCs and found that FGF9 significantly enhanced the proliferation (Fig. 5G), amount of the population (Fig. 5H) and sphere formation (Fig. 5I) of CSCs, while treatment with FGF receptor inhibitor, PD173074, significantly suppressed these properties of CSCs (Fig. 5G, 5H, 5I). To further examine the effect of PDGF-BB, we introduced shPDGFB into TAM. Similarly, we introduced shRNA to both FGF7 and FGF9 into BM-hMSC cells. These cells and CSCs were then co-injected into mouse tibiae. We found that the knockdown of PDGFB in TAM indeed significantly abrogated the tumor growth promoting effect of TAM in the bone (Fig. 5J). Furthermore, knockdown of FGF7 and FGF9 in BM-hMSC also significantly suppressed growth promoting effect of BM-hMSC (Fig. 5K).

We consider that Task 1 is accomplished.

Task 2. To test whether the activated TAM can promote recurrence in the animal model
  (a) Examine the localization of TAM in recurrent mouse model.
  (b) Test whether TAM induces recurrence of dormant breast cancer cells *in vivo.*

We have established the colony of MMTV-rtTA and TetO-ErbB2 and we also successfully obtained MMTV-rtTA+/- /TetO-ErbB2+/- heterozygous. That was enough to conduct Task 1 experiment; however, to accomplish Task 2, we need more number of animals and this cross-breeding is quite inefficient. Therefore, we have establish homozygous mouse for both genes (MMTV-rtTA+/+, and TetO-ErbB2+/+). The cross-breeding of these mice will yield desired phenotype of offspring with 100% efficiency. While we were breeding these mice, we needed to re-locate our institution from SIU School of Medicine to University of Mississippi Medical Center and our breeding effort was temporarily suspended due to the transfer of these animals to UMMC and to obtain an approval for a new animal protocol. This unexpected move significantly delayed our progress on Task 2; however, we have now received the approval and the breeding is currently underway at UMMC.

Task 2 is incomplete and ongoing.

KEY RESEARCH ACCOMPLISHMENTS

1. We have isolated CSCs from breast cancer and shown that they indeed have a strong tumor initiating ability as well as metastatic capability.
2. We have shown that CM of cancer cells is necessary to convert monocytes to tumor associate macrophage.
3. In our animal model, we have shown that TAM significantly promoted the growth of CSCs in the bone *in vivo*.
4. We have shown that TAM secreted PDGF after interacting with CSCs, which then activated the stromal cells to secrete FGF7 and 9.
5. The FGF7 and 9 significantly promoted proliferation of CSCs.
6. We have established mouse colonies for MMTV-rtTA and TetO-ErbB2. The cross-breeding is ongoing.

REPORTABLE OUTCOMES

Peer reviewed publications
 None

Abstract/presentation

1. Aya Kobayashi, Hiroshi Okuda, Fei Xing, Wen Liu, Puspa Pandey, Misako Watabe, Sudha K. Pai, and Kounosuke Watabe. The role of tumor associated macrophage in recurrent growth of tumor stem cell in bone metastasis. Department of Defense (DOD) Breast Cancer Research Program (BCRP) Era of Hope 2011 Conference. Orlando, Florida

Employment

1. Aya Kobayashi, Ph.D. (postdoctoral fellow) has been partly supported by the current grant.
2. Koji Fukuda, Ph.D.(postdoctoral fellow) was also partly supported by this grant.
3. Mr. Ravindra Kirki (Graduate student) has been partly supported by the grant.

CONCLUSIONS
We have established necessary mouse colonies and also developed the method to generate TAM. We have also shown that TAM indeed promoted the growth of CSCs in our animal model. Due to the relocation of our entire lab, Task 2 which involves extensive animal breeding has been delayed; however, we have compensated the lack of progress in Task 2 by further extending our mechanistic study in Task 1. We have shown that the interaction of TAM with CSCs lead to enhanced secretion of PDGF-BB from TAMs which then activated stromal cells and enhanced CSC self-renewal.

So what?
The pro-inflammatory microenvironment in the tumor is established by first recruiting various leukocytes including tumor-associated macrophage (TAM) which is believed to promote tumor growth and angiogenesis, suppress adaptive immunity, and hence remodel tumor microenvironment (4). Therefore, understanding the underlining mechanism of tumor-TAM interaction in cancer recurrence is of paramount interest for developing a novel approach to treat and prevent recurrent breast cancer. We

believe that the outcome of our results set a new paradigm in our understanding of metastatic niche of CSCs in the bone, which may provide novel targets such as TAM, PDGF and FGF, for the treatment of recurrent disease.

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